Three photoconvertible forms of green fluorescent protein identified by spectral hole-burning

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Several studies have led to the conclusion that, in the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a photoconversion involving excited-state proton transfer occurs from an A- to a B-form, while an intermediate I-form was held responsible for the green fluorescence. Here we have identified the I-form of wild-type GFP in absorption, located the 0-0 transitions of all three forms A, B and I, and determined vibrational frequencies of the ground and excited states. The intrinsically narrow 0-0 transitions are revealed by the wavelengths at which holes can be burnt. The pathways of photointerconversion are unraveled by excitation, emission and hole-burning spectroscopy. We present an energy-level scheme that has significant implications for GFP-mutants, which likewise can occur in the three photointerconvertible forms.

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has triggered a revolution in molecular biology as the only cloned protein to exhibit strong green fluorescence without cofactors[1]. When this small single-chain protein consisting of 238 amino acids[2] is fused to other proteins, it serves as a marker for gene expression and allows visualization of dynamic events inside the living cell[3,4]. The crystal structure of GFP has been solved[5,6], and the chromophore results from an autocatalytic cyclization and subsequent oxidation of the three residues Ser 65, Tyr 66 and Gly 67[7–9]. The room temperature absorption spectrum of wild-type GFP (wt-GFP) is characterized by two maxima (Fig. 1), which have been attributed to different protonation states of the chromophore: the maximum at ~398 nm to a neutral A-form and that at ~478 nm to an anionic or deprotonated B-form[10–14] or, alternatively, to a cation (A) and a zwitterion (B)[15].

Boxer and coworkers[10] examined the excited-state dynamics of the chromophore at room temperature and at 77 K and concluded that the interconversion from A to B involves an excited-state proton transfer and passes through an excited intermediate state I*. They proposed that it is I* that gives rise to the strong fluorescence at 508 nm[8,12,16] and that it can evolve further to B* (ref. 10). In this model, I* and I (the latter not identified spectroscopically) were unrelaxed forms of B* and B[10,11]. The details of the photoconversion, however, remained unclear. We have solved the pathways of photoconversion for wt-GFP and observed the I-form in the ground state by using a combination of optical spectroscopic methods at both room temperature and low temperature (1.6 K).

As noted before[10,11], the absorption spectrum at 295 K narrows upon cooling (Fig. 1). At 1.6 K it is more structured, and the ratio of the absorbances of the A- and B-forms inverts with respect to

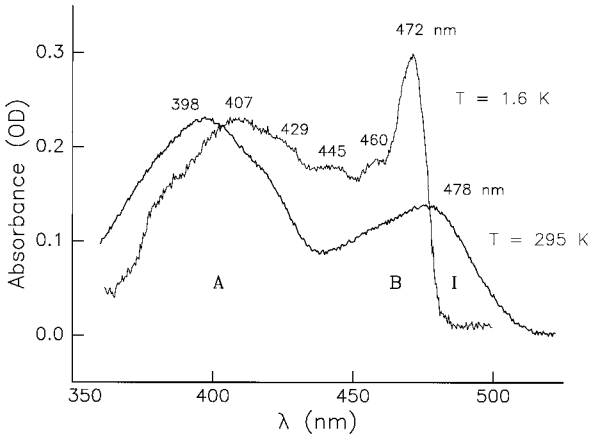

Fig. 1 Absorption spectra of wild-type green fluorescent protein (GFP) at room temperature (T = 295 K) and low temperature (T = 1.6 K). The red wing of the spectrum at 295 K arises from the I form.

that at 295 K, indicating that B has a slightly lower ground-state energy than A. The remarkable changes in the red edge of the spectrum are particularly relevant. While the 478 nm maximum at 295 K shifts to 472 nm at 1.6 K, the broad wing on the red side of this maximum disappears at 1.6 K. In light of the results presented here, we assign this red wing to the I-form, which has thus far escaped detection in the ground state. Because the I-form is populated at 295 K, but is absent at 1.6 K, its ground-state energy must be somewhat higher than that of A and B.

To prove our assignment, we first compare the excitation and emission spectra at 295 K and 1.6 K (Fig. 2). At room temperature (Fig. 2*a*), they intersect at ~495 nm; at 1.6 K (Fig. 2*b*), they do so at 477 nm. If only forms A and B were present in the ground state, one would not expect such an appreciable shift with temperature. The results support the hypothesis that a third, intermediate I-form contributes to the spectra at room temperature. The spectra at 295 K (Fig. 2*a*) further show that on excitation into the A form at 400 nm there is a weak emission from A in the 440–460 region, in addition to the strong green fluorescence at 508 nm characteristic for wt-GFP. The emission spectrum at 295 K corroborates the suggestion[10,11] that the excited state A* rapidly photoconverts into I*, which then emits at 508 nm. The maxima marked in the excitation spectrum of Fig. 2, bottom, correspond to vibronic bands of B* and A*, and those of the emission spectra to vibronic bands of B (see also Fig. 3). The bands of B and B* are mirror symmetrical about its 0-0 transition with frequencies of 220, 770 and 1510 cm^{-1}; the Stokes shift, given by half the difference between the first maximum in excitation (472 nm) and in emission (482 nm), is $\Delta = (220 \pm 10)\ cm^{-1}$.

The remarkable variation of the emission and excitation spectra at 1.6 K with the excitation (λ_{exc}) and detection (λ_{det}) wavelengths (Fig. 3) is a further indication of the role played by the I form. At $\lambda_{exc} \leq 435$ nm, both the A and B forms are excited; thus the weak emission bands in the 440–460 nm region, which disappear for $\lambda_{exc} \geq 435$ nm, must belong to vibronic bands of A. Therefore, we expect the 0-0 transition (origin) of A to lie in the 430–435 nm region. When exciting into the A form at $\lambda_{exc} \leq 435$ nm, the process A*→I* occurs and the emission spectra exhibit strong fluorescence from I*. For $\lambda_{exc} \geq 435$ nm, only the B form

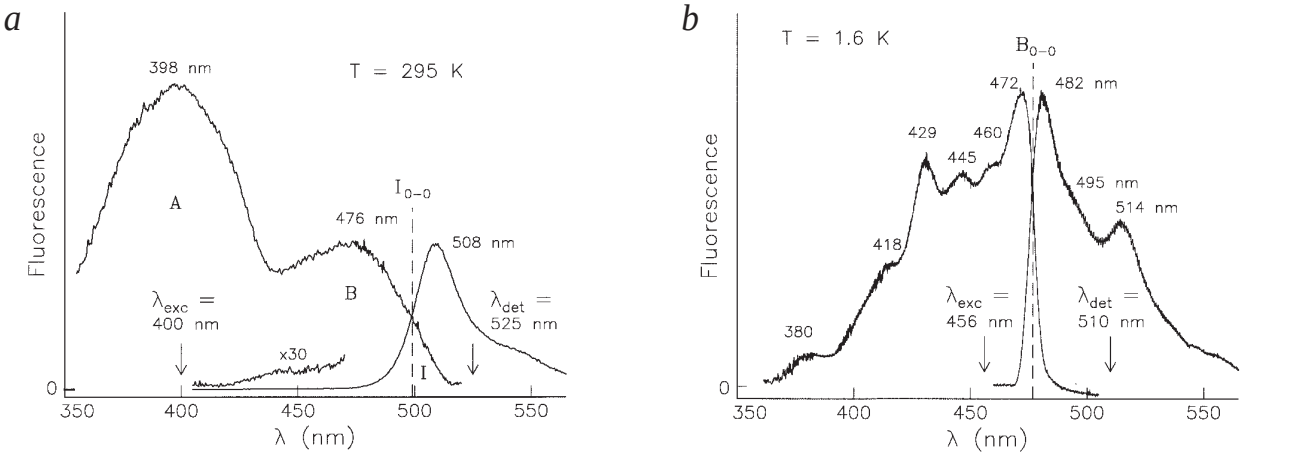

Fig. 2 Excitation and fluorescence spectra of GFP at ***a***, 295 K and ***b***, 1.6 K. The intersection of the red wing of the excitation spectrum and the blue wing of the fluorescence spectrum marks the wavelength of the 0-0 transition of the I form at room temperature (*a*) and of the B form at low temperature (*b*).

is excited and emits light, but no B*→I* conversion takes place. The two emission spectra excited at 456 and 477 nm (top of Fig. 3*a*) are almost identical. They are only slightly shifted with respect to each other, implying that the 0-0 region of B, in which fluorescence line-narrowing occurs, must be in the 475–480 nm region (Fig. 4). The excitation spectra (Fig. 3*b*) show vibronic bands of A* and B*, but not of I*, in agreement with our suggestion that the I form is not thermally populated at 1.6 K.

Illumination of the A form (Fig. 4*a*) with a laser at 400–430 nm causes selective bleaching of the A form with the simultaneous emergence of a new band at 490–495 nm, which we assign to the I form. The emission spectrum obtained on excitation into this I band at 493 nm (Fig. 4*b*) is very similar to the corresponding part of the emission spectrum obtained on excitation into the A form (Fig. 3*a*). Thus by burning into A at 1.6 K, a stable I form is produced in the ground state, most probably through the conversion A*→I*→I. When burning into B, no emission from I* is observed; nevertheless I is produced in its ground state through a radiationless conversion B*→I. The I form generated in this way has an identical emission spectrum as that obtained after burning into A (Fig. 4*d*). Inversely, when I is burnt (in the region 490–495 nm), it photoconverts by a radiationless process back into A and B (results not shown). At 1.6 K, in contrast to room temperature, it is not possible to phototransform A↔B directly, but only through the intermediate I.

Attempts to burn narrow spectral holes[17] at arbitrary wavelengths in the absorption bands at 1.6 K proved fruitless. We discovered, however, that there are exceptions in the regions where the 0-0 transitions were anticipated. By selective excitation into the A-form within the range (434 ± 1) nm, narrow holes can be burnt. Because it is not possible to burn narrow holes at any other wavelength within the absorption region of A, we conclude that A_{0-0} lies at (434 ± 1) nm. Since vibronic transitions decay much faster than 0-0 transitions (picoseconds *versus* nanoseconds), they yield much broader holes[17]. Likewise, we have located the I_{0-0} transition at (495 ± 1) nm and the B_{0-0} transition at (477 ± 1) nm by hole-burning. Furthermore, on burning a deep 0-0 hole, an additional broad phonon side-hole, red-shifted by $\delta = 50–60$ cm^{-1}, always appears. With this value and that of the Stokes shift $\Delta = (220 \pm 10)$ cm^{-1}, we obtain an electron–phonon coupling strength $S = \Delta/\delta = 4.0 \pm 0.5$. This large coupling explains why it is so difficult to burn holes in wt-GFP and to determine the 0-0 transitions. Note that the observed hole widths are limited by the laser bandwidth of ~1

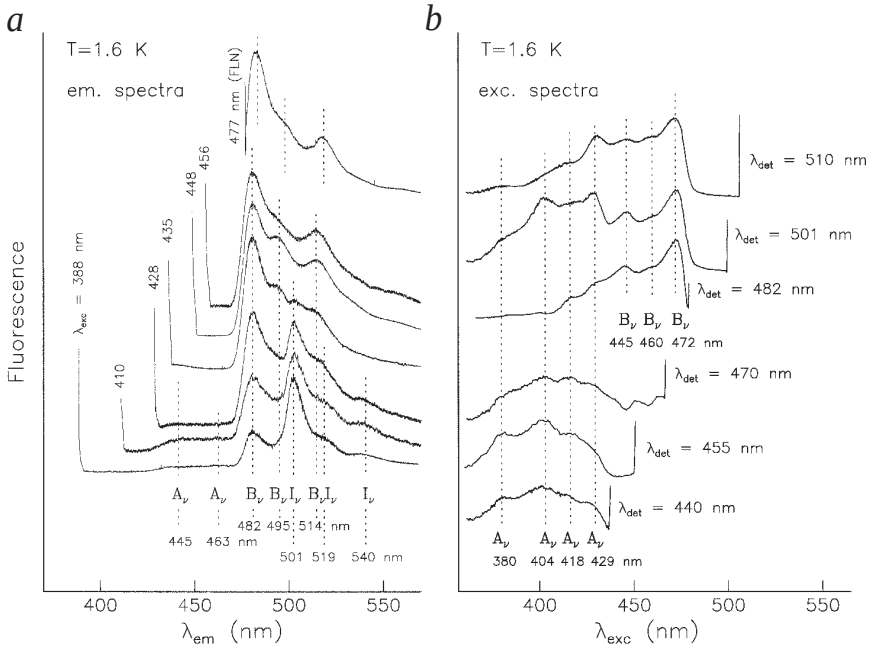

Fig. 3 ***a***, Emission and ***b***, excitation spectra of GFP at 1.6 K recorded at various excitation and emission wavelengths, respectively. The features in the spectra correspond to vibrations of the ground state (*a*) and electronically excited state (*b*) of the three forms A, B and I.

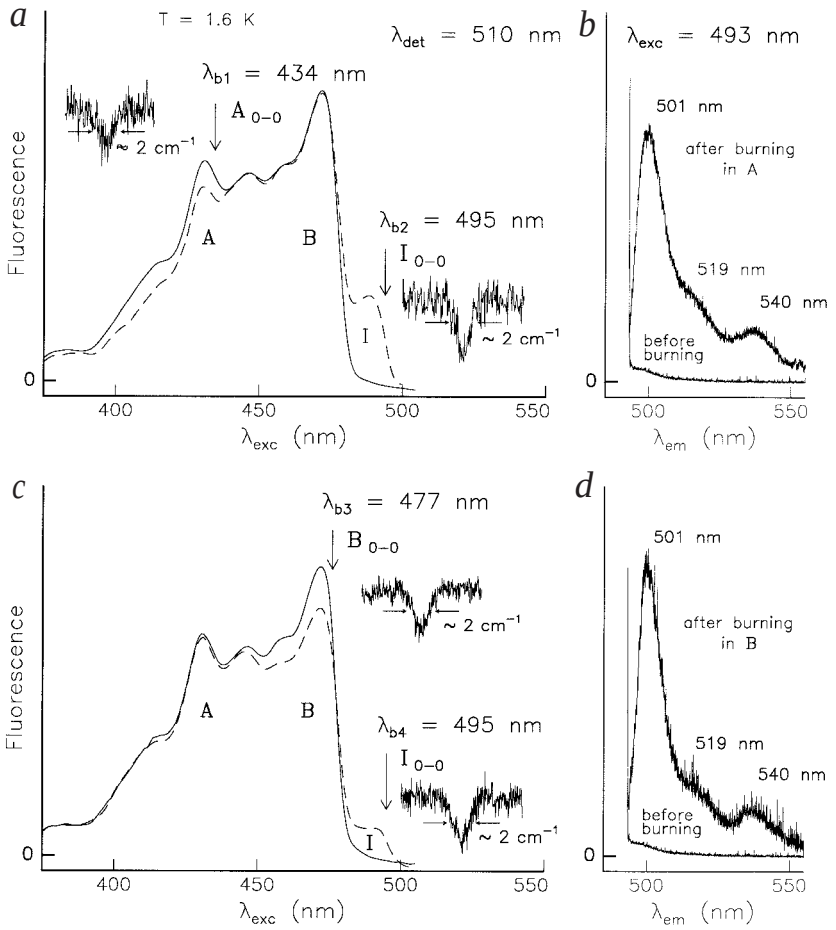

Fig. 4 Excitation spectra before (——) and after (– – –) burning (*a*, *c*, λ_{det} = 510 nm), and corresponding emission spectra (*b*,*d*, λ_{exc} = 493 nm) of GFP at 1.6 K. ***a***, Burning into A produces I. The 0-0 transitions of A and I are found by hole-burning at (434 ± 1) nm and (495 ± 1) nm, respectively. The widths of the holes of ~2 cm^{-1} are limited by twice the laser bandwidth. ***b***, Emission spectrum of I upon direct excitation of I. The photoinduced reaction between A and I, and B and I are reversible. ***c***, Burning into B produces I. The 0-0 transition of B is found at (477 ± 1) nm; that of I again at (495 ± 1) nm. ***d***, Emission spectrum of I upon direct excitation of I. The photoinduced reaction between B and I is reversible.

cm^{-1} and are not representative of the homogeneous linewidth[17].

Knowing the 0-0 transitions, we can establish the photointerconversion pathways in an energy-level diagram (Fig. 5). Since the ground state of I is not populated at 1.6 K and is only weakly populated at room temperature, it must lie above that of B and A by a few hundred cm^{-1}. I, when populated by burning into either A or B at 1.6 K, remains populated upon warming to 150 K, but tends to the low thermal equilibrium value at 295 K, suggesting that the height of the barriers in the ground state separating A and B from I must also be of the order of hundreds of wavenumbers. This picture is supported by bleaching experiments at room temperature: when B is photoinduced by burning into A, it slowly reverts (in hours) to thermal equilibrium. In the excited state, the barrier for the process A*→I* appears to be low. In contrast, the barrier for B*→I* should be at least 2,000 cm^{-1} since excitation into B*, even in its vibronic bands, does not induce any emission from I*, but only produces I in the ground state. The conversions B*→I, I*→B and I*→A all occur through radiationless processes.

To confirm that the red wing in the absorption spectrum at 295 K of Fig. 1 results from population in the ground state of I, we have excited at ~495 nm at room temperature and verified that the maximum of the emission indeed occurs at 508 nm, the same wavelength as that observed on excitation of the A form at 400 nm (Fig. 2*a*). When exciting B at 465–470 nm, however, the emission maximum is shifted to the blue to 490 nm. We thus conclude that the I-form is indeed populated at room temperature. The results show that optical switching between conformations of GFP is feasible in bulk samples at both low and high temperature and is not restricted to single-molecule experiments[18]. Having identified the 0-0 transitions, we can determine their homogeneous linewidths and gain insight into dynamical processes such as chromophore–protein and protein–protein interactions in these three conformations.

GFP has been extensively mutated to modify and improve its fluorescence properties[3,6,8,16]. For red-shifted mutants, we have found similar results as for wt-GFP (T.M.H. Creemers *et al.*,in preparation). Although thermal equilibrium at room temperature strongly favors one of the three forms A, I or B in these mutants, this form can still be photointerconverted into the others. This phenomenon may have important consequences when using GFP mutants as fusion tags or markers in cells and organisms. For example, an observed change in the emission spectrum arising from an intramolecular photointerconversion may be erroneously attributed to dynamic intermolecular processes occurring with-

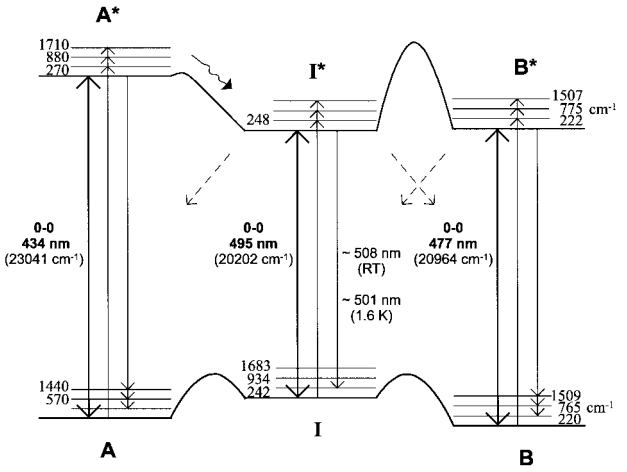

Fig. 5 Proposed energy-level scheme of the three photoconvertible forms A, I and B of wt-GFP. The 0-0 and vibronic transitions (in cm^{-1}) are given. The dashed diagonal arrows indicate photoinduced radiationless conversions. The only photoconversion between excited states in wt-GFP is that from A* to I *.

in the cell.

Methods

Samples. Recombinant wild-type *Aequorea victoria* GFP incorporating an N-terminal six-Histidine tag was expressed in *Escherichia coli* and purified. All spectroscopic measurements were performed with ~40 μM protein dissolved in 10 mM sodium phosphate buffer, pH 7.0, containing 50% glycerol at 295 K and 1.6 K. For low-temperature measurements, the sample was placed in a cuvette (diameter 3 mm) and introduced into an empty ^{4}He-bath cryostat that had first been filled with liquid N_2. The latter was blown out after a few minutes; the cryostat was then filled with liquid He and pumped down to 1.6 K.

Absorption, excitation, emission and hole-burning spectroscopy. The optical experiments were carried out by exciting the sample with a dye-laser (Molectron DL 200, bandwidth ~1 cm^{-1}) pumped by a pulsed-N_2 laser (Molectron UV 22). The dye-laser was continuously tunable from 360 to 515 nm. Absorption spectra were recorded in transmission through the sample and detected with a photomultiplier (type EMI 9658 B). Emission, excitation and hole-burning spectra were detected in fluorescence at 90° with respect to the excitation beam through a 0.85 m-double monochromator (SPEX 1402, resolution 5 cm^{-1}) with the same photomultiplier.

Acknowledgments

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Revealing the structure of the oxygen-evolving core dimer of photosystem II by cryoelectron crystallography

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Here we present cryoelectron crystallographic analysis of an isolated dimeric oxygen-evolving complex of photosystem II (at a resolution of ~0.9 nm), revealing that the D1–D2 reaction center (RC) proteins are centrally located between the chlorophyll-binding proteins, CP43 and CP47. This conclusion supports the hypothesis that photosystems I and II have similar structural features and share a common evolutionary origin. Additional density connecting the two halves of the dimer, which was not observed in a recently described CP47–RC complex that did not include CP43, may be attributed to the small subunits that are involved in regulating secondary electron transfer, such as PsbH. These subunits are possibly also required for stabilization of the dimeric photosystem II complex. This complex, containing at least 29 transmembrane helices in its asymmetric unit, represents one of the largest membrane protein complexes studied at this resolution.

Photosystem II (PSII) is a multisubunit, membrane protein complex that catalyzes the light-driven water-splitting reaction of photosynthesis[1]. In so doing, it releases oxygen into the atmosphere and produces the reducing equivalents necessary to convert carbon dioxide to organic compounds. Despite the biological importance of PSII, its high-resolution structure has not yet been determined. The redox-active reaction center (RC) of PSII is composed of the D1 and D2 proteins, which are functionally and structurally related to the L and M subunits of the purple bacteria reaction center for which there are high-resolution structures[2]. Closely associated with the D1 and D2 proteins are two chlorophyll-binding proteins, CP43 and CP47(inner antenna proteins), which, together with the 33 kDa extrinsic protein, a cluster of four manganese atoms and additional extrinsic and low molecular weight proteins, form the oxygen-evolving core complex[1]. *In vivo*, this PSII core complex is coupled to a number of Lhcb proteins that bind chlorophyll *a* and *b* and form an outer light-harvesting antenna.

Like the bacterial L and M subunits, the D1 and D2 proteins of PSII have five transmembrane helices each and together form a heterodimer[3] (Fig. 1). CP43 and CP47 are predicted to consist of six transmembrane helices[4], making a total of 22 transmembrane helices for the CP47–CP43–D1–D2 assembly (Fig. 1). The reaction center of PSI is composed of two similar proteins (PsaA and PsaB), which together also have a total of 22 transmembrane helices (Fig. 1). The 4 Å structure of PSI has been determined by X-ray crystallography[5,6] revealing that the two sets of 11 transmembrane helices of the PSI PsaA and PsaB proteins are related by pseudo two-fold symmetry. Notably, the five C-terminal transmembrane helices of both the PsaA and PsaB proteins are organized in a manner comparable to that of the five helices of the bacterial L and M subunits and also the D1 and D2 helices of PSII (Fig. 1). Furthermore, it has been suggested that the six N-terminal transmembrane helices of the PSI PsaA and PsaB proteins may be similar in structure to the six helices of CP43 and CP47